A Pooled Analysis of the Prognostic and Predictive Effects of KRAS Mutation Status and KRAS Mutation Subtype in Early Stage Resected Non-small Cell Lung Cancer in Four Trials of Adjuvant Chemotherapy

Shepherd, et al

Supplemental Material for On-line Publication Only

Supplemental Methods

KRAS mutation analyses

In JBR.10, *RAS* mutational status was a stratification variable, and was analyzed prospectively. For all other trials, *KRAS* analyses were performed after completion of the clinical studies. In JBR.10, *RAS* mutation analysis for codons 12, 13 and 61 of *H-, K-* and *N-RAS* was performed by Allelic Specific Oligonucleotide Hybridization with all mutations confirmed by sequencing. Full details have been published previously[1].

For IALT[2] and ANITA, *KRAS* mutation analysis was performed using PCR amplification and direct sequencing of exon 2, which contains mutation ‘‘hotspots’’ at codons 12 and 13. For some ANITA cases that failed direct sequencing, the TheraScreen®: K-RAS Mutation Kit (DxS, Manchester, UK) was used. Additional confirmation was performed using the Restriction Length Fragment Polymorphism (RLFP) method.[3] Tumor rich areas with estimated >30% tumor cellularity were macrodissected from paraffin-embedded archived tissue sections and genomic DNA was extracted using a standard QIAamp DNA extraction Kit (Qiagen, Hilde, Germany). The *KRAS* gene was amplified with AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) and with primers 1F 5' ACTGAATATAAACTTGTGGTAGTTGGAGCT-3' and 3R 5' GGTGCAGGACCATTCTTTGATACAGAT-3'. The PCR reaction contained 1.5 mM $MgCl_2$, 0.4 µM of each primer, 200 µM of each dNTP, 1.5 U of *Taq*, 10 mM Tris-HCl and 50 mM KCl. The *Taq* was activated by incubation at 95^{o}C for 3 min. The PCR reaction consisted of 50 cycles (94^{o}C for 30 sec, 55^{o}C for 30 sec and 72^{o}C for 30 sec) followed by a final extension reaction at 72^{o}C for 10 min, generating a 157 bp

amplification product which was sequenced by bidirectional automated sequencing (ABI PRISM BigDye Terminator v1.1 Cycle sequencing, Applied Biosystems, Foster City, CA).

For CALGB-9633, *KRAS* exon 2 was amplified using the following PCR primers: forward : 5'-GTGTATTAACCTTATGTGTGAC-3' Reverse :5'-CTGTATCAAAGAATGGTCCTGCA-3'. The PCR amplification was performed using 53ºC annealing temperature and generated a 237 bp product. The resulting PCR product was digested for 20 minutes at 42C using the Surveyor^{TM} enzyme (Transgenomic) using previously established methods[3]. The resulting products were separated using a bioanalyzer (Agilent). DNA from H23 and H441 cells were used as a positive control. All samples that produced digestion products, indicative of a mutation or polymorphism, were verified to be mutations using sequencing.

References

1. Tsao M.-S, Liu N, Nicklee T, Shepherd F, Viallet J. Angiogenesis correlates with vascular endothelial growth factor expression but not with Ki-ras oncogene activation in non-small cell lung cancer. *Clin Cancer Res* 3:1807-1814, 1997.

2. Ma X, Vataire AL, Sun H, Dunant A, Brambilla E, Soria J, Filipits M, Hainaut P. TP53 and KRAS mutations as markers of outcome of adjuvant cisplatin-based chemotherapy in completely resected non-small-cell lung cancer: The International Adjuvant Lung cancer Trial (IALT) biological program. 33rd ESMO Congress, Stockholm, September 2008. *Ann Oncol* 2008 (19, Suppl 8): viii 61.

3. Soulières D, Greer W, Magliocco AM, Huntsman D, Young S, Tsao MS, Kamel-Reid S. KRAS mutation testing in the treatment of metastatic colorectal cancer with anti-EGFR therapies. *Curr Oncol*. Suppl 1:S31-40, 2012.

4. Jänne PA, Borras AM, Kuang Y, Rogers AM, Joshi VA, Liyanage H, Lindeman N, Lee JC, Halmos B, Maher EA, Distel RJ, Meyerson M, and Johnson B.E. A rapid and sensitive enzymatic method for EGFR mutation screening. *Clin Cancer Res* 12:751-8, 2006.

Statistical analyses

A Cox proportional hazards regression model was used to evaluate the association between *KRAS* status and time dependent endpoints, for instance overall survival. The following covariates were included: treatment, sex, age, PS, tumor-stage, nodal-stage and histology. The global strategy to build the multivariable Cox model included 2 steps. The first step was to study the correlation between *KRAS* status and other covariates. The second step was to study the association between covariates and overall survival. The covariates which were significant in the first or the second step were included in the final multivariable Cox model. Treatment and *KRAS* status also were included in this model.

Step 1: the first analysis examined the correlation between *KRAS* status and other covariates (sex, age, PS, smoking status, Stage, N, T, histology and type of surgery) using a univariate logistic model stratified by trial. The results of this analysis are presented in the Table 1 of the paper. All covariates significantly associated with *KRAS* status ($p<0.20$) were included in the multivariable logistic model stratified by trial. Only age and histology remained significantly associated with *KRAS* status ($p<0.05$). These 2 covariates were included in the final Cox model.

Step 2: the second analysis consisted of a univariate analysis to evaluate the association between the covariates and survival (Supplemental Table 8). All

covariates except smoking status then were included in a multivariable Cox model since the p-values (univariate analysis) were $\leq$0.20. Stage was not included in the model due to its correlation with T-stage and N-stage. The hazards ratios of the multivariable Cox model are reported in Supplemental Table 9. The covariates sex, PS, N-stage, T-stage and histology remained significantly associated with overall survival ($p<0.05$) after adjustment and were included in the final Cox model with KRAS and treatment (Supplemental Table 10).

Supplemental On-line Table 1
Patient and tumor characteristics for patients with and without *KRAS* mutation results*

		Patients *KRAS* status unknown** (N=514)		Patients *KRAS* status known (N=1543)		p-value ***
		N	%	N	%	
Trial	*ANITA*	93	*18*	110	*7*	<0.0001
	IALT	311	*61*	718	*47*	
	JBR.10	32	*6*	450	*29*	
	CALGB	78	*15*	265	*17*	
Radiotherapy	*No*	349	*68*	1280	*83*	0.005
	Yes	165	*32*	263	*17*	
Sex	*Male*	418	*81*	1152	*75*	0.51
	Female	96	*19*	391	*25*	
Age category	*< 50*	100	*19*	231	*15*	0.10
	50-59	177	*34*	513	*33*	
	60-69	187	*36*	603	*39*	
	>=70	50	*10*	196	*13*	
WHO performance	*0*	275	*53*	810	*52*	0.22
Status	*1*	199	*39*	668	*43*	
	2	36	*7*	61	*4*	
	Unknown	4	*1*	4	*<1*	
Smoking status	*No*	18	*4*	68	*4*	0.07
	Yes	182	*35*	754	*49*	
	*Unknown****	314	*61*	721	*47*	
Stage	*Stage IA*	20	*4*	69	*4*	0.15
	Stage IB	194	*38*	696	*45*	
	Stage II	162	*32*	526	*34*	
	Stage III	136	*26*	247	*16*	
	Unknown	2	*<1*	5	*<1*	
Histology	*Squamous cell*	239	*46*	707	*46*	0.06
	Adenocarcinoma	208	*40*	605	*39*	
	Other NSCLC	62	*12*	231	*15*	
	Unknown	5	*1*	0	*0*	
Type of surgery	*Pneumonectomy*	155	*30*	464	*30*	0.08
	Other	356	*69*	1076	*70*	
	Unknown	3	*1*	3	*<1*	
Treatment arm	*OBS*	254	*49*	763	*49*	0.96
	ACT	260	*51*	780	*51*	

ACT, adjuvant chemotherapy; OBS, observation; WHO, World Health Organization
* Patients only from centers participating in specimen collection for correlative studies (202/406 centers)
** The patients without *KRAS* status include patients without tumor tissue samples and patients without results after *KRAS* analysis
*** Chi-square test from a logistic model stratified by trial. Patients with unknown values were excluded from the corresponding analyses.
**** No tobacco-related data collected in IALT trial

Supplemental On-line Table 2
Patient and tumor characteristics by trial for 1543 patients with *KRAS* results

		ANITA (n=110) n *(%)*	IALT (n=718) n *(%)*	JBR.10 (n=450) n *(%)*	CALGB (n=265) n *(%)*
Radiotherapy planned	*No*	64 *(58)*	501 *(70)*	450 *(100)*	265 *(100)*
	Yes	46 *(42)*	217 *(30)*	0	0
Sex	*Male*	103 *(94)*	582 *(81)*	293 *(65)*	174 *(66)*
	Female	7 *(6)*	136 *(19)*	157 *(35)*	91 *(34)*
Age category	*< 50*	17 *(15)*	108 *(15)*	64 *(14)*	42 *(16)*
	50-59	33 *(30)*	260 *(36)*	138 *(31)*	82 *(31)*
	60-69	51 *(47)*	287 *(40)*	180 *(40)*	85 *(32)*
	≥ 70	9 *(8)*	63 *(9)*	68 *(15)*	56 *(21)*
WHO performance status	*0*	47 *(43)*	395 *(55)*	220 *(49)*	148 *(56)*
	1	61 *(55)*	264 *(37)*	229 *(51)*	114 *(43)*
	2	2 *(2)*	59 *(8)*	0	0
	Unknown	0	0	1 *(< 1)*	3 *(1)*
Smoking status*	*No*	7 *(6)*	0	51 *(11)*	0
	Yes	103 *(94)*	0	399 *(89)*	0
	Unknown	0	718 *(100)*	0	265 *(100)*
Stage	*Stage IA*	1 *(1)*	65 *(9)*	0	3 *(1)*
	Stage IB	46 *(42)*	190 *(26)*	208 *(46)*	252 *(95)*
	Stage II	33 *(30)*	248 *(35)*	242 *(54)*	3 *(1)*
	Stage III	30 *(27)*	215 *(30)*	0	2 *(1)*
	Unknown	0	0	0	5 *(2)*
Histology	*Squamous cell carcinoma*	56 *(51)*	408 *(57)*	156 *(35)*	87 *(33)*
	Adenocarcinoma	33 *(30)*	223 *(31)*	210 *(47)*	139 *(52)*
	Other NSCLC	21 *(19)*	87 *(12)*	84 *(18)*	39 *(15)*
Type of surgery	*Pneumonectomy*	39 *(35)*	291 *(41)*	105 *(23)*	29 *(11)*
	Other	71 *(65)*	427 *(59)*	345 *(77)*	233 *(88)*
	Unknown	0	0	0	3 *(1)*

WHO, World Health Organization
* No tobacco-related data collected in IALT trial

Supplemental Online Table 3
***Number of patients (%) according to KRAS* mutation nucleotide* changes**

		G>T	G>A, G>T**	G>C	G>A
Codon 12	ANITA (n=20)	14 (70.0%)	1 (5.0%)	5 (25.0%)	0 (0.0%)
	IALT (n=90)	71 (78.9%)	0 (0.0%)	3 (3.3%)	16 (17.8%)
	JBR10 (n=105)	75 (71.4%)	2 (1.9%)	11 (10.5%)	17 (16.2%)
	CALGB (n=60)	50 (83.3%)	0 (0.0%)	3 (5.0%)	7 (11.7%)
	TOTAL	210	3	22	40
Codon 13	ANITA (n=2)	2 (100%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	IALT (n=7)	5 (71.4%)	0 (0.0%)	1 (14.3%)	1 (14.3%)
	JBR10 (n=6)	1 (16.7%)	0 (0.0%)	1 (16.7%)	4 (66.7%)
	CALGB (n=9)	5 (55.6%)	0 (0.0%)	0 (0.0%)	4 (44.4%)
	TOTAL	13	0	2	9

* Patients with mutation in codon are 14 not included (one patient)
** Double mutation
Nucleotides: G, (guanine); A, (adenine); C, (cytosine); T, (thymine)

Supplemental Online Table 4
Number of patients (%) according to KRAS mutation amino acid substitutions*

		G12A	G12C	G12D, G12C**	G12R	G12V	G12D, G12V***	G13C	G12D	G12S	G13D	G13R
Codon 12	ANITA (n=20)	4 (20.0%)	11 (55.0%)	1 (5.0%)	2 (10.0%)	2 (10.0%)	0	0	0	0	0	0
	IALT (n=90)	1 (1.1%)	46 (51.1%)	0	1 (1.1%)	25 (27.8%)	0	0	11 (12.2%)	6 (6.7%)	0	0
	JBR10 (n=105)	8 (7.6%)	50 (47.6%)	0	3 (2.9%)	25 (23.8%)	2 (1.9%)	0	14 (13.3%)	3 (2.9%)	0	0
	CALGB (n=60)	3 (5.0%)	38 (63.3%)	0	0	12 (20.0%)	0	0	6 (10.0%)	1 (1.7%)	0	0
	TOTAL	16	145	1	6	64	2	0	31	10	0	0
Codon 13	ANITA (n=2)	0	0	0	0	0	0	2 (100.0%)	0	0	0	0
	IALT (n=7)	0	0	0	0	0	0	5 (71.4%)	0	0	1 (14.3%)	1 (14.3%)
	JBR10 (n=6)	0	0	0	0	0	0	1 (16.7%)	0	0	4 (66.7%)	1 (16.7%)
	CALGB (n=9)	0	0	0	0	0	0	5 (55.6%)	0	0	4 (44.4%)	0
	TOTAL	0	0	0	0	0	0	13	0	0	9	2

* Amino acids: G (glycine), A (alanine), C (cystine), D (aspartic acid), R (arginine), S (serine), V (valine)

** Patient with mutation on codon 14 is not included

*** Double mutation

Supplemental Online Table 5
KRAS mutations by smoking status*

Smoker status	*KRAS* mutations				Total
	G>T	G>A, G>T	G>C	G>A	
No	5 (3.4%)	0	2 (10%)	4 (12.5%)	11
Yes	142 (96.6%)	3	18 (90%)	28 (87.5%)	191
Total	147	3	20	32	202

*Analyzed only in ANITA, JBR10, and CALGB trials as smoking status not collected in IALT.

Supplemental Online Table 6
KRAS codon 12 or codon 13 mutations by smoking status*

Smoker status	*KRAS* codon	*KRAS* mutations			
		G>T	G>A, G>T	G>C	G>A
No	**12**	5 (2.5%)	0	2 (1%)	3 (1.5%)
	13	0	0	0	1 (0.5%)
Yes	**12**	134 (66.3%)	3 (1.5%)	17 (8.4%)	21 (10.4%)
	13	8 (4.0%)	0	1 (0.5%)	7 (3.5%)

*Analyzed only in ANITA, JBR10, and CALGB trials as smoking status not collected in IALT

Supplemental On-line Table 7
KRAS mutations at codon 12: amino acid substitutions and smoking status*

Smoker status	Amino acid substitutions			Total
	G12C or G12V	G12D or G12S	G12A or G12R	
No	5 (3.6%)	3 (12.5%)	2 (10%)	10
Yes	133 (96.4%)	21 (87.5%)	18 (90%)	175
Total	138	24	20	185**

* Data only for ANITA, JBR10, and CALGB trial as data on smoking status not collected in IALT ** The three patients (two from JBR10 and one from ANITA) with double mutations on codon 12 were excluded from the analysis according to amino acid substitution and were smokers.

Supplemental On-line Table 8
Association of baseline characteristics and overall survival: univariate analysis

Univariate analysis (1,543)		N	%	HR [95%CI]	p-value*
Sex	*Male*	1152	*74.7*	1	< 0.0001
	Female	391	*25.3*	0.65 [0.54-0.78]	
Age	*< 55*	432	*28.0*	1	0.05
	55-64	628	*40.7*	1.20 [1.00-1.43]	(0.02)
	≥ 65	483	*31.3*	1.26 [1.04-1.52]	
WHO performance	*0*	810	*52.5*	1	0.001
Status	*1 / 2*	729	*47.2*	1.27 [1.10-1.47]	
	Unknown	4	*0.3*		
Smoking status**	*No*	58	*3.8*	1	0.36
	Yes	502	*32.5*	0.83 [0.55-1.24]	
	Unknown	983	*63.7*		
Stage	*I*	765	*49.6*	1	< 0.0001
	II	526	*34.1*	1.73 [1.44-2.09]	(< 0.0001)
	III	247	*16.0*	2.93 [2.35-3.64]	
	Unknown	5	*0.3*		
N-Stage***	*0*	859	*55.7*	1	< 0.0001
	1	477	*30.9*	1.57 [1.31-1.88]	(< 0.0001)
	2	199	*12.9*	2.83 [2.28-3.51]	
	Unknown	8	*0.5*		
T-Stage***	*1*	174	*11.3*	1	< 0.0001
	2	1164	*75.4*	1.27 [0.99-1.64]	(< 0.0001)
	3 / 4	200	*13.0*	2.04 [1.52-2.72]	
	Unknown	5	*0.3*		
Histology	*Squamous cell*	707	*45.8*	1	0.13
	Adenocarcinoma	605	*39.2*	1.03 [0.87-1.20]	
	Other NSCLC	231	*15.0*	1.23 [1.00-1.52]	
Type of surgery	*Pneumonectomy*	464	*30.1*	1	< 0.0001
	Other	1076	*69.7*	0.74 [0.63-0.86]	
	Unknown	3	*0.2*		

HR, Hazard Ratio, CI: confidence interval
* p-values are calculated from Wald test (test for trend).
** No tobacco-related data collected in IALT trial (718 patients); this variable was not included in the multivariable models
*** 6th Edition TNM Staging classification

Supplemental On-line Table 9
Association of baseline characteristics and overall survival: multivariable analysis

Multivariable analysis (n=1,529)┼		HR [95%CI]	p-value
Sex	*Male*	1	< 0.0001
	Female	0.66 [0.54-0.80]	
Age	*< 55*	1	0.06
	55-64	1.15 [0.95-1.38]	
	≥ 65	1.26 [1.04-1.54]	
WHO performance status	*0*	1	0.03
	1 / 2	1.18 [1.02-1.36]	
N-Stage*	*0*	1	< 0.0001
	1	1.60 [1.32-1.93]	
	2	2.78 [2.22-3.47]	
T-Stage*	*1*	1	< 0.0001
	2	1.27 [0.99-1.65]	
	3 / 4	1.97 [1.43-2.70]	
Histology	*Squamous cell*	1	0.0012
	Adenocarcinoma	1.29 [1.08-1.54]	
	Other NSCLC	1.43 [1.15-1.77]	
Type of surgery	*Pneumonectomy*	1	0.65
	Other	0.96 [0.80-1.15]	

HR, Hazard Ratio
┼ patients with missing covariate are excluded of the analysis
* 6th Edition TNM Staging classification

Supplemental On-line Table 10
Association of baseline characteristics and overall survival: multivariable analysis with *KRAS*

Multivariable analysis (n=1,531)	KRAS mutation	Comparison	HR [95%CI]	p-value**
Sex		Female *vs.* Male	0.67 [0.55-0.81]	< 0.0001
Age				0.05$^{\mu}$
		55-64 *vs.* < 55	1.15 [0.96-1.38]	0.14
		≥ 65 *vs.* < 55	1.27 [1.05-1.55]	0.02
WHO performance status		PS1/2 *vs.* P0	1.17 [1.01-1.36]	0.03
N-Stage*				<0.0001$^{\mu}$
		N1 *vs.* N0	1.59 [1.32-1.92]	< 0.0001
		N2 *vs.* N0	2.81 [2.26-3.49]	< 0.0001
T-Stage*				<0.0001$^{\mu}$
		T2 *vs.* T1	1.27 [0.98-1.63]	0.07
		T3/4 *vs.* T1	2.01 [1.49-2.73]	< 0.0001
Histology				0.006$^{\mu}$
		Adeno *vs.* Squamous	1.21 [1.01-1.45]	0.04
		Other *vs.* Squamous	1.39 [1.12-1.72]	0.003
Codon				0.86$^{\mu}$
		Codon 12 *vs.* WT	1.08 [0.82-1.44]	0.58
		Codon 13 *vs.* WT	1.03 [0.48-2.19]	0.94
***KRAS* mutation**				0.004$^{\mu}$
	Wild-Type	ACT *vs.* OBS	0.89 [0.76-1.04]	0.15
	Codon 12	ACT *vs.* OBS	0.95 [0.67-1.35]	0.77
	Codon 13	ACT *vs.* OBS	5.78 [2.06-16.22]	0.001

HR, Hazard Ratio; CI, Confidence Interval
* 6th Edition TNM Staging classification
** Wald test p-values
$^{\mu}$ overall test for the covariate

Supplemental Online Table 11
Predictive value of *KRAS* on overall survival by histology

	ACT arm (No deaths / No patients)	OBS arm (No deaths / No patients)	Hazard ratio for death CT vs. no CT [95% CI]
Squamous cell carcinoma Hazard ratio for death mutated vs. Wild-type [95% CI]	1.46 [(a)] [0.76;2.78] p = 0.25	1.10 [(b)] [0.58;2.10] p = 0.76	**Test for interaction *KRAS**treatment p=0.55**
***KRAS* Wild-type status n=662**	162 / 342	163 / 320	0.90 [0.72 ; 1.12] p=0.34
***KRAS* Mutated status n=43**	10 / 17	10 / 26	1.19 [0.49 ; 2.87] p=0.70
Adenocarcinoma Hazard ratio for death mutated vs. Wild-type [95% CI]	1.02 [(a)] [0.71;1.46] p = 0.92	0.98 [(b)] [0.70;1.38] p = 0.89	**Test for interaction *KRAS**treatment p=0.86**
***KRAS* Wild-type status n=396**	86 / 190	102 / 206	0.88 [0.66 ; 1.17] p=0.37
***KRAS* Mutated status n=203**	47 / 104	48 / 99	0.92 [0.61; 1.37] p=0.67
Other NSCLC Hazard ratio for death mutated vs. Wild-type [95% CI]	2.29 [(a)] [1.33;3.93] p = 0.003	1.36 [(b)] [0.70;2.67] p = 0.36	**Test for interaction *KRAS**treatment p=0.24**
***KRAS* Wild-type status n=176**	47 / 91	43 / 85	0.90 [0.59 ; 1.36] p=0.62
***KRAS* Mutated status n=52**	19/ 32	11 / 20	1.51 [0.71 ; 3.20] p=0.28

ACT, adjuvant chemotherapy; OBS, observation

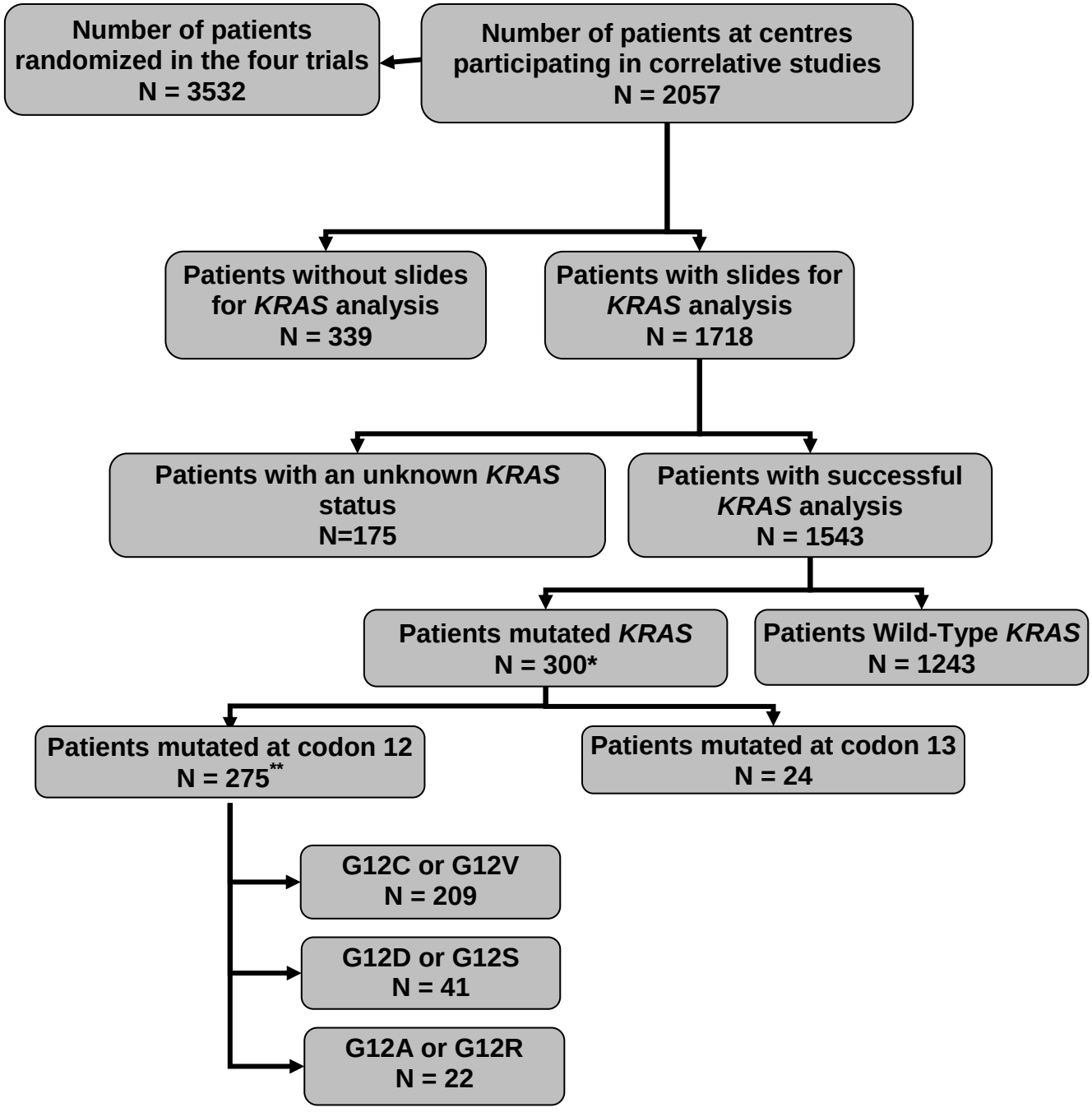
a) The three HRs in the ACT arm are significantly different: test of heterogeneity p=0.02.

b) The three HRs in the OBS arm are not significantly different: test of heterogeneity p=0.44.

The interaction between histology, KRAS status and treatment is not significant, p=0.53

Supplemental On-line Figure 1

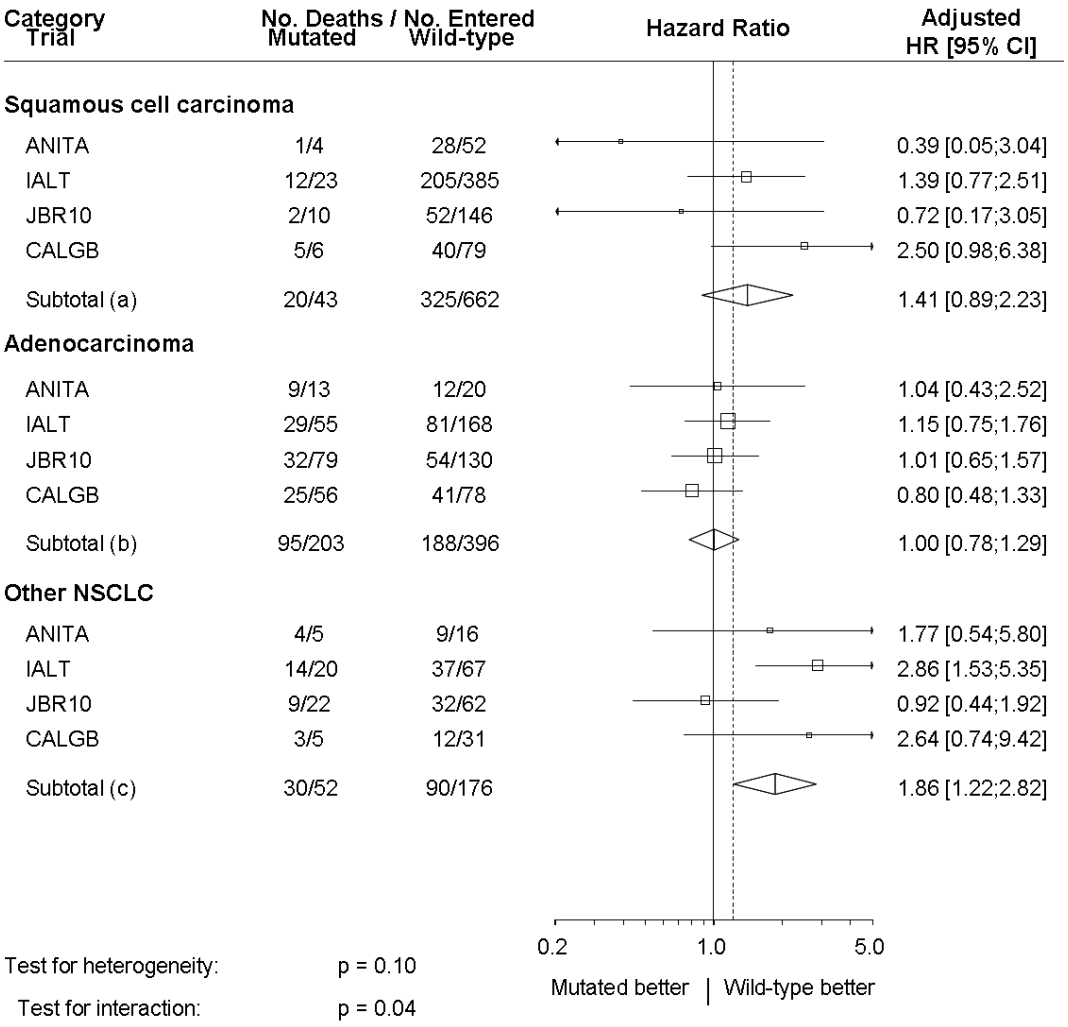
Flow chart of patients entered in the four trials and patients in the final *KRAS* analyses



* One patient had a rare mutation at codon 14. This patient is included in all analyses of KRAS mutation versus wild-type, but is excluded from mutation subtype analyses.

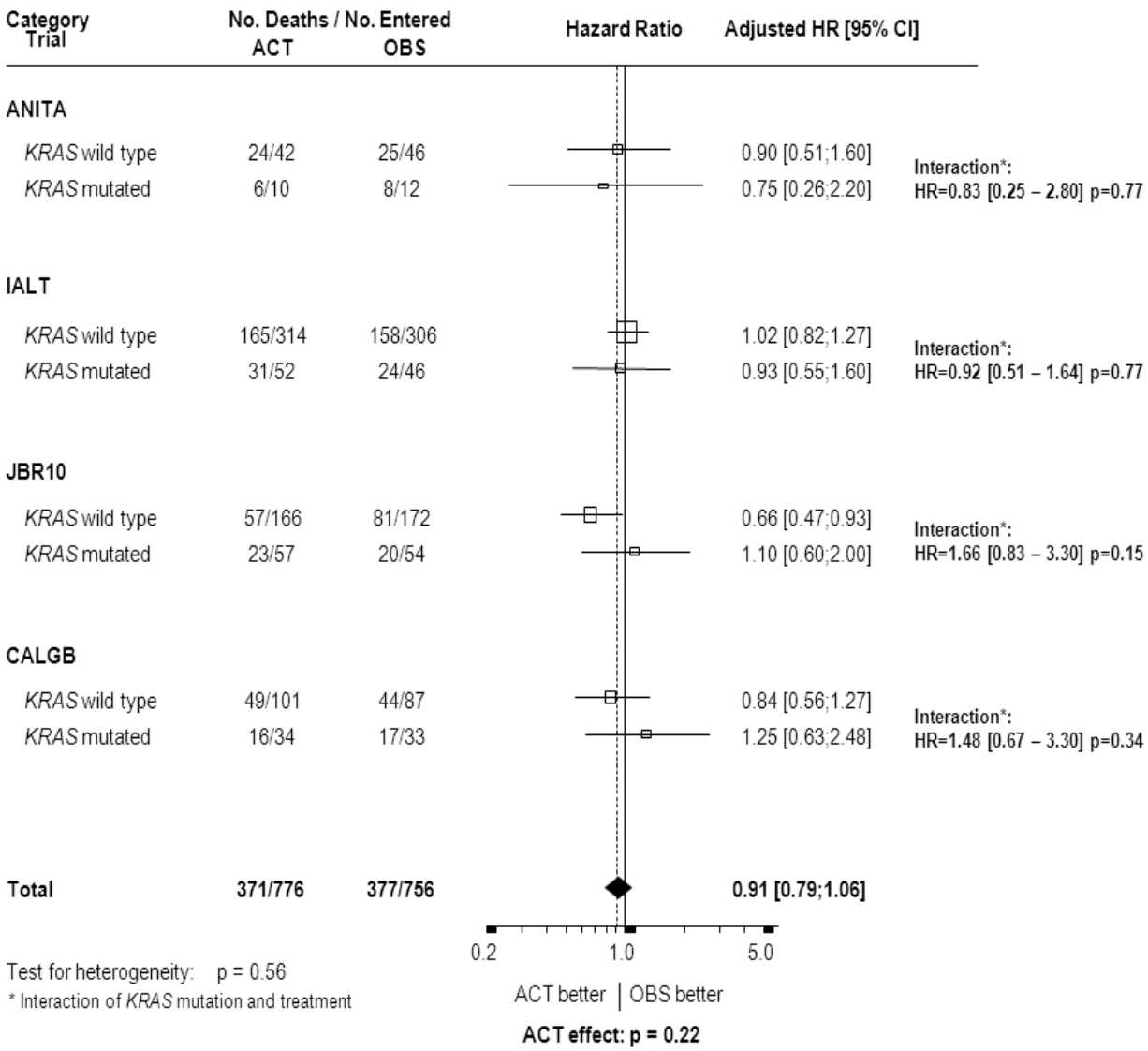
**3 patients with double mutation on codon 12 are excluded from the analysis according to amino acid substitution.

Supplemental Online Figure 2
Prognostic value of *KRAS* on overall survival by histology and trial



Note: The heterogeneity test compares the 12 hazard ratios by histology and by trial; the test for interaction compares the three subtotal hazard ratios.

Supplemental Online Figure 3
Predictive value of *KRAS* on overall survival by trial



ACT, adjuvant chemotherapy; OBS, observation

Note: The heterogeneity test compares the 8 hazard ratios by *KRAS* status and trial. The p-value of the test of equality of the four hazard ratios of interactions between *KRAS* and treatment is p=0.52.